



Virginia Commonwealth University
VCU Scholars Compass

Theses and Dissertations


Graduate School

2008

Regulation of Platelet-Activating Factor Acetylhydrolase by Oxidized Phospholipids and Proinflammatory Cytokines

Muralikrishna Mukkamala
Virginia Commonwealth University

Follow this and additional works at: <http://scholarscompass.vcu.edu/etd>

 Part of the [Biochemistry, Biophysics, and Structural Biology Commons](#)

© The Author

Downloaded from

<http://scholarscompass.vcu.edu/etd/653>

This Thesis is brought to you for free and open access by the Graduate School at VCU Scholars Compass. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of VCU Scholars Compass. For more information, please contact libcompass@vcu.edu.

© Muralikrishna Mukkamala, 2008

All Rights Reserved

REGULATION OF PLATELET-ACTIVATING FACTOR ACETYLHYDROLASE BY
OXIDIZED PHOSPHOLIPIDS AND PROINFLAMMATORY CYTOKINES

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of
Science in Biochemistry and Molecular Biology at Virginia Commonwealth University.

by

MURALIKRISHNA MUKKAMALA
B.A., George Washington University, 2005

Director: SUZANNE E. BARBOUR, PH.D.
PROFESSOR
DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

Virginia Commonwealth University
Richmond, Virginia
May 2008

Acknowledgement

I would like to acknowledge those people who helped me throughout my graduate studies. First and foremost, I want to thank Dr. Suzanne E. Barbour for giving me a position in her laboratory and teaching me practically everything I know about biochemical research. I would like to thank Rachael Griffiths, who like Dr. Barbour has shown me several techniques and furthermore, is always there with a helping hand. I must also acknowledge the heavy contribution she has had on this thesis and my project as a whole. I would like to thank the members of my committee, Dr. Xianjun Fang and Dr. Daniel H. Conrad for their support and advice throughout the final stages of my graduate education. I want to thank Megan Price for her help with the ELISA analysis of IL-6 and sharing her data with us. I would like to thank Dr. Norbert Leitinger for collaborating with our lab and providing us with the oxidized lipids, which are absolutely essential for our research. I want to thank Dr. Alexandre Fabiato, for he more than anyone else raised my interest and awareness of cardiovascular physiology. And finally, I would like to thank Dr. Tomasz Kordula for playing tennis with me, for had he not done so, I would have received next to no physical exercise.

Table of Contents

	Page
Acknowledgements.....	ii
List of Figures	iv
Abstract	v
Chapter	
1 Introduction.....	1
2 Hypothesis.....	11
3 Methods.....	12
4 Results.....	15
5 Discussion	33
6 Literature Cited	39

List of Figures

	Page
Figure 1: The development of an atherosclerotic plaque in the wall of artery	2
Figure 2: Migration and differentiation of monocytes in the arterial intima and large foam cell formation	3
Figure 3: Mechanism of PAFAH activity	6
Figure 4: Differential response in dendritic cells and macrophages	8
Figure 5: The PAFAH 5' Flanking Region has a GC box and a GAS sequence.	10
Figure 6: IL-6 Induces PAFAH in Dendritic Cells	16
Figure 7: IL-6 Induces PAFAH Activity in Dendritic Cells, but not in Macrophages	18
Figure 8: IL-8 Induces PAFAH in Dendritic Cells	20
Figure 9: IL-8 Induces PAFAH Activity in Dendritic Cells, but not in Macrophages	22
Figure 10: The IL-6 Receptor is more highly expressed in dendritic cells than in macrophages	24
Figure 11: OxPAPC-induced PAFAH Expression and Activity follow a lag	26
Figure 12: OxPAPC-induces secretion of IL-6 in Dendritic Cells	28
Figure 13: OxPAPC treatment activates Stat3 in Dendritic Cells	30
Figure 14: Time-course showing elevation of PAFAH in response to IL-6	32
Figure 15: Time-course showing changes in IL-6 secretion, Stat3 activation, and PAFAH expression and activity	35
Figure 16: Theoretical Model	36

Abstract

REGULATION OF PLATELET-ACTIVATING FACTOR ACETYLHYDROLASE BY OXIDIZED PHOSPHOLIPIDS AND PROINFLAMMATORY CYTOKINES

By Muralikrishna Mukkamala, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Biochemistry and Molecular Biology at Virginia Commonwealth University.

Virginia Commonwealth University, 2008

Major Director: Suzanne E. Barbour, Ph.D.
Professor, Department of Biochemistry and Molecular Biology

Platelet-Activating Factor Acetylhydrolase (PAFAH) is a monocyte-derived phospholipase A₂ that catalyzes the hydrolysis of platelet-activating factor (PAF) and has been implicated in atherosclerosis. Although PAF and other proinflammatory stimuli are postulated to induce the enzyme, mechanisms controlling PAFAH expression are largely unknown at present. We have shown that PAFAH induction in monocytes is increased in response to oxidized phospholipids. The PAFAH 5' flanking region has at least 10 putative Stat elements, and IL-6 has been shown to be downstream from the prostaglandin receptor, EP2, which has been shown to bind oxidized phospholipids, prompting the hypothesis that

Stat proteins might regulate its expression. To test this hypothesis, we treated human monocytes with IL-6, a monocyte-derived cytokine that activates Stat3, IL-8, a monocyte-derived cytokine induced by Stat3, and oxidized 1-palmitoyl-2-arachidonoyl-*sn*-3-phosphocholine (oxPAPC), a major component of the oxidized LDL particle. Two monocyte-derived cell types, macrophages (MO) and dendritic cells (DC) were prepared from primary human monocytes. The cells were treated with various doses of IL-6, IL-8, or oxPAPC for various time frames in the absence of serum. Culture supernatants from the cytokine-treated cells were harvested and screened for PAFAH protein and activity and cell monolayers were assessed for PAFAH mRNA by quantitative real time PCR (qPCR). Cells treated with oxPAPC were further analyzed for secreted IL-6 using ELISA and activation of Stat3 using Western Blot. Both IL-6 and IL-8 induced PAFAH expression in a dose-dependent manner. Although both MO and DC responded to the cytokines, preliminary experiments suggested that induction of PAFAH is more robust in DC than MO. Cytokine-treated cells exhibited increased PAFAH activity in their culture supernatants that correlated with increased PAFAH protein levels. Treatment with oxPAPC induced IL-6 secretion and subsequent Stat3 activation in DC. Together, these data support the hypothesis that PAFAH expression is regulated by oxidized phospholipids and proinflammatory cytokines in developing atheromas.

Introduction

Atherosclerosis is a chronic inflammatory condition affecting arterial blood vessels. The chronic inflammatory response is due to the buildup of low-density lipoprotein (LDL) in the arterial intima, where it can be oxidized by a variety of different cellular processes or free radicals. In response, white blood cells then migrate into the arterial wall. They begin to adhere to the walls of the artery and develop into large fatty foam cells. As these cells continue to accumulate, a fatty streak is formed. Eventually, necrosis and subsequent fibrosis may occur in the streak, causing development of a plaque, which may finally rupture and cause formation of an occlusive blood clot (Fig 1).

The buildup of LDL in the arterial intima and its subsequent oxidation serves as a chemo-attractant for monocytes, which is what causes these cells to migrate into the arterial intima. There, they may differentiate into other monocyte-derived cell types such as dendritic cells or macrophages. Through the action of their scavenger receptors, they uptake large amounts of oxidized LDL and develop into large foam cells, which adhere to the walls of the artery and contribute to the developing atheroma (Fig 2) (1).

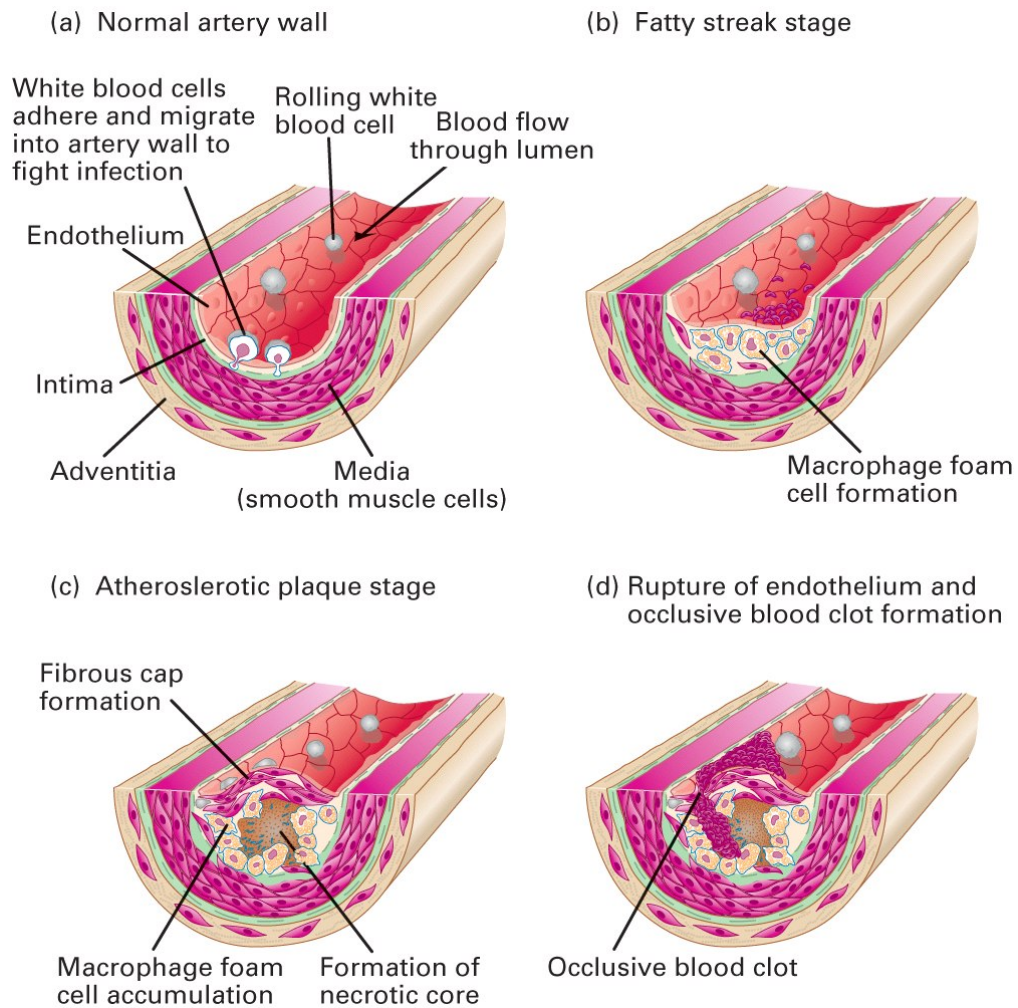
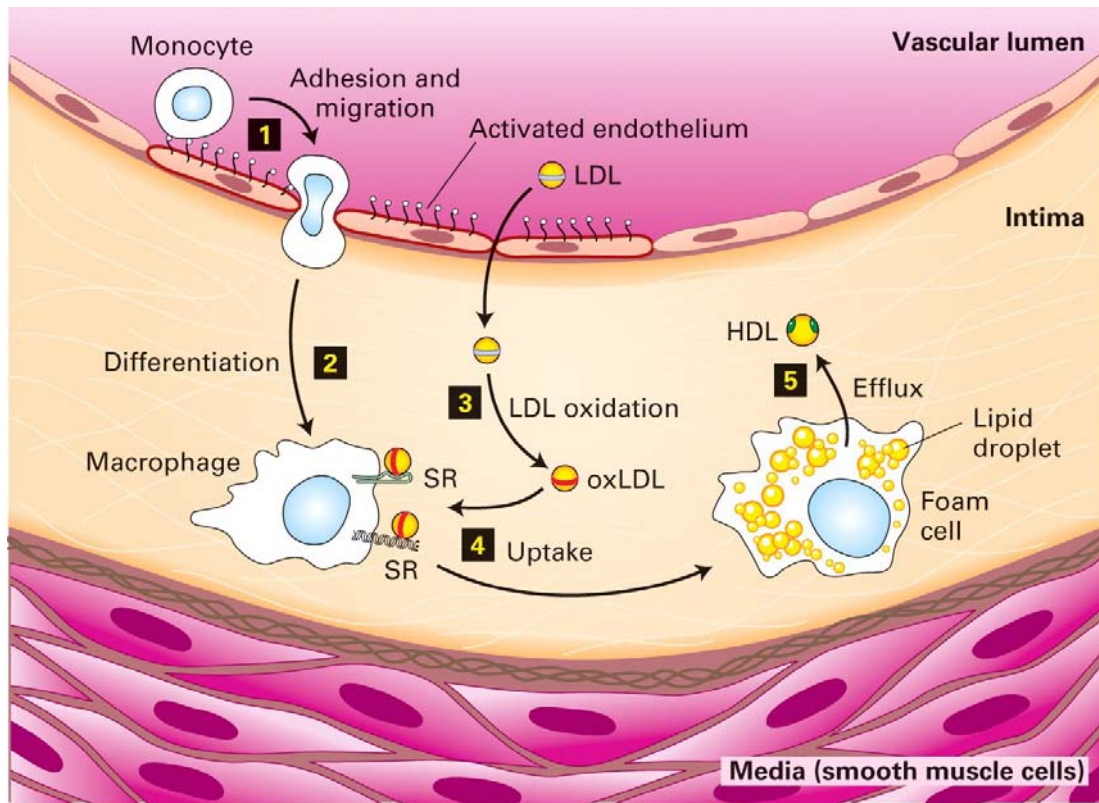


Figure 1: The development of an atherosclerotic plaque in the wall of artery. Lodish et al, 2004, p. 768.



(a)

Figure 2: Migration and differentiation of monocytes in the arterial intima and large foam cell formation. Lodish et al, 2004, 769.

The LDL particle can become trapped and build up in the arterial intima and then become oxidized (2). The core of the LDL particle is comprised of triglycerides, cholesteryl esters, and free cholesterol. However, the surface of the LDL particle is dominated by oxidized phospholipids. It is these oxidized phospholipids that are some of the agents responsible for signaling in atherosclerosis (3). OxPAPC (oxidized 1-palmitoyl-2-arachidonoyl-*sn*-3-phosphocholine) is a major component of the oxidized LDL particle. Upon oxidation, other modified phosphatidylcholines become present that have oxidatively fragmented or modified *sn*-2 acyl chains. Among the major components of oxPAPC are 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-3-phosphocholine (POVPC), 1-palmitoyl-2-(5,6-epoxyisoprostane E₂)-*sn*-3-phosphocholine (PEIPC), and 1-palmitoyl-2-glutaroyl-*sn*-3-phosphocholine (PGPC) (4). It has been shown that the long chain fractions, such as PEIPC, as opposed to the short chain fractions, such as PGPC and POVPC, are the bioactive lipids that cause signaling in developing atheromas (5). In addition, the moiety present on the *sn*-2 position of PEIPC bears a similar structure to that of a prostaglandin. Theoretically, this may play a role in its ability to bind receptors involved in developing atheromas.

In addition to acting as a chemo-attractant and inducing the recruitment of monocytes to the arterial intima, oxidized phospholipids have been shown to induce secretion of proinflammatory cytokines that exacerbate atherosclerosis. In particular, studies have shown that plasma concentrations of Interleukin-6 (IL-6) and Interleukin-8 (IL-8) are elevated in patients with atherosclerosis (6;7).

The enzyme Platelet-Activating Factor Acetylhydrolase (PAFAH) has also been shown to be upregulated in atherosclerosis. PAFAH is a secreted, 45kDa, calcium-independent phospholipase A₂ that catalyzes the hydrolysis of platelet activating factor (PAF) and other short-chain or oxidatively fragmented phospholipids at the *sn*-2 position (Fig 3). PAFAH can associate with lipoproteins and is also referred to as the lipoprotein-associated phospholipase A₂. In general, phospholipase A₂ is known to be an upstream regulator of inflammatory responses. Upon cleaving phospholipids at the *sn*-2 position, arachidonic acid and lysophospholipids are released, which can then be modified into various inflammatory mediators, such as prostaglandins or leukotrienes. PAFAH differs from other types of phospholipase A₂ in that it is calcium independent and secreted (8).

Monocytes are the major source of secreted PAFAH in the circulation, and it has been shown that expression of PAFAH increases greatly when monocytes differentiate into macrophages, and to a lesser extent, dendritic cells (9;10).

PAFAH is implicated in atherosclerosis, and its presence correlates with the severity of the disease. However, its role is controversial with some studies suggesting protection but others indicating that the enzyme exacerbates disease. One study showed that Japanese subjects with a mutation nullifying PAFAH activity had a higher rate of heart attack and atherosclerosis. However, another study showed that in European men, the same loss of function mutation correlated with a reduced risk of heart attack or coronary artery disease (10-15). While it is clear that PAFAH plays a role in atherosclerosis, few studies have addressed the regulation of its expression and activity in atherosclerotic lesions.

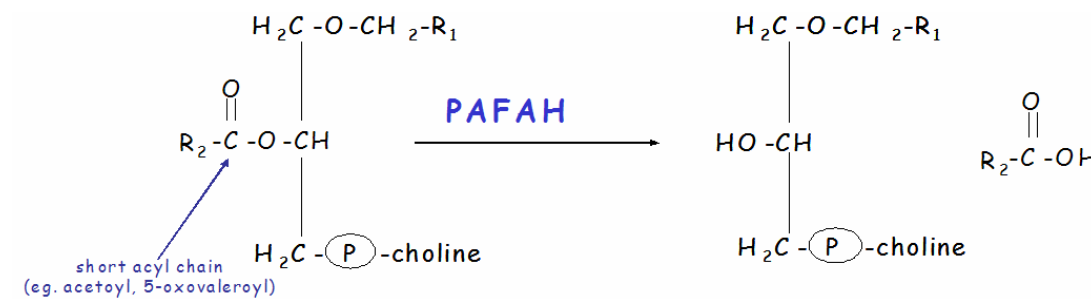


Figure 3: Mechanism of PAFAH activity. PAFAH selectively hydrolyzes PAF and other short-chain phospholipids at the *sn*-2 position, catabolizing them, but also releasing bioactive lipids.

It has previously been shown that oxidized 1-palmitoyl-2-arachidonoyl-*sn*-3-phosphocholine (oxPAPC), a major component of oxidized LDL, can bind the prostaglandin EP2 receptor (3;5;16;17). Our lab has also shown that PAFAH induction is increased in response to oxPAPC in dendritic cells, but not macrophages (Fig 4).

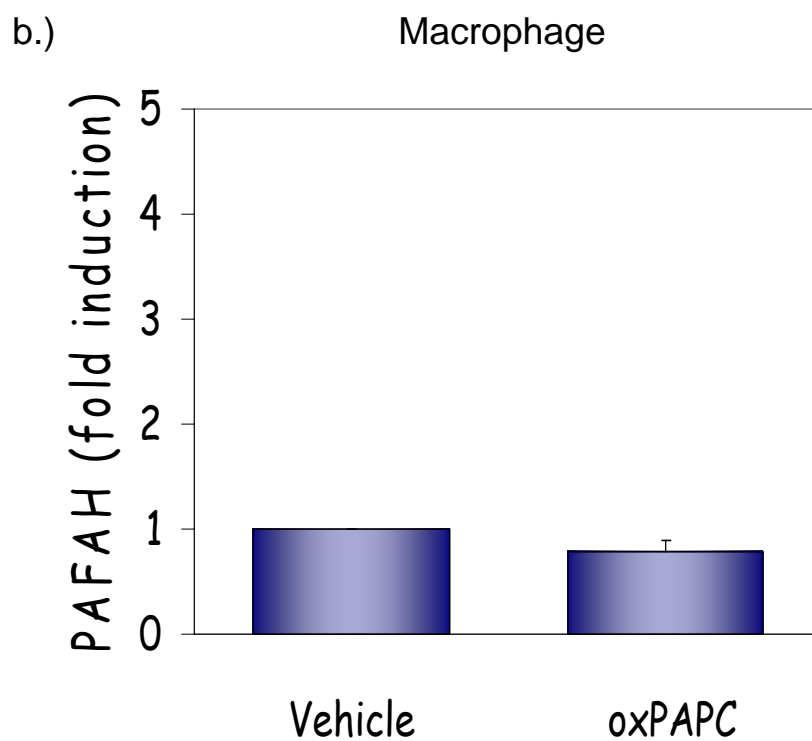
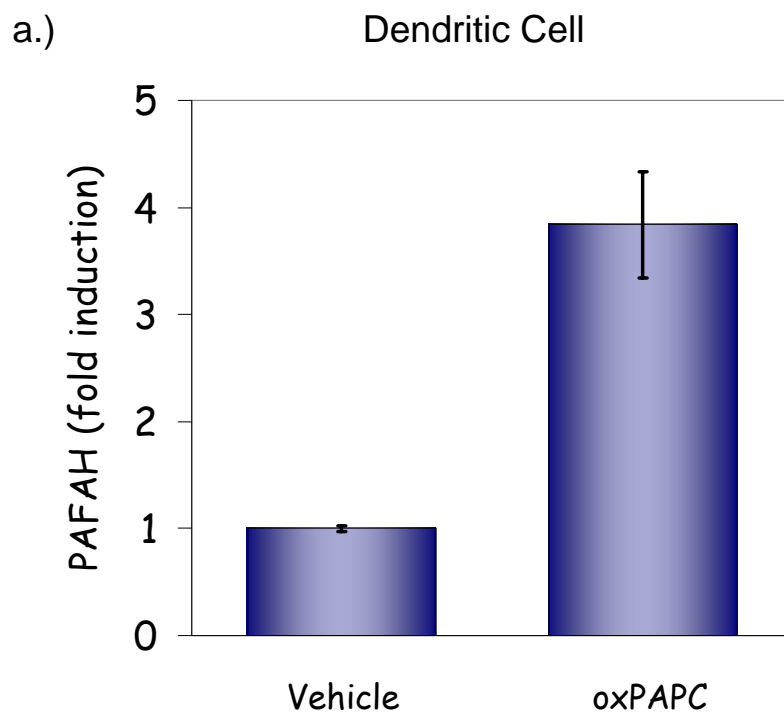


Figure 4: Differential response in dendritic cells and macrophages. PAFAH induction is increased in response to oxPAPC in a.) dendritic cells, but not b.) macrophages.

It has previously been shown that lipopolysaccharide (LPS) strongly induces PAFAH expression. LPS is a classical stimulus of inflammation, suggesting that the upregulation of PAFAH is also mediated similarly. In addition, the transcription factors, Sp1 and Sp3 are required for both LPS-induced expression as well as basal expression. Sp1 and Sp3 bind to a GC-rich box in the 5' flanking region of the PAFAH gene, however the relative levels of Sp1 and Sp3 are not increased during LPS-stimulated PAFAH induction, suggesting another factor may be involved (18-20). In the 5' flanking region, there also exists an interferon-gamma activated sequence (GAS), a classic target of Stat transcription factors. Because EP2 has been previously shown to be involved in oxPAPC signaling, and that IL-6 is downstream of EP2 (21;22), IL-6 activation of Stat3 and its subsequent activation of PAFAH could be a novel mechanism illustrating the induction of PAFAH by oxidized phospholipids in atherosclerosis.

In addition, it has been shown in endothelial cells that IL-8 induction is increased in response to oxidized phospholipids in a Stat3 dependant mechanism (22). This suggests that IL-8 could also be involved in the induction of PAFAH by oxidized phospholipids and proinflammatory cytokines.

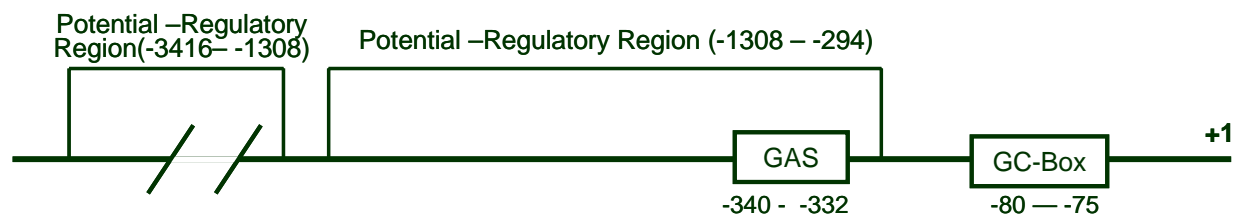


Figure 5: The PAFAH 5' Flanking Region has a GC box and a GAS sequence. Sp1 and Sp3 are required for basal expression, and bind the GC box. The GAS sequence is a classic target for Stat3 binding, thus driving PAFAH expression.

Hypothesis

We hypothesize that in atherosclerosis, oxPAPC induces dendritic cells to express increased levels of PAFAH by binding the EP2 receptor. This causes an increase in IL-6 secretion which autocrinely activates the cell of origin or paracrinely activates adjacent dendritic cells. IL-6 then activates Stat3, which drives PAFAH expression and secretion. Subsequently, Stat3 drives IL-8 expression which then augments the PAFAH response through an unknown mechanism.

Methods

Isolation of Monocytes and Differentiation into Dendritic Cells and Macrophages:

Peripheral blood leukocytes were extracted from human blood using lymphocyte separation media (LSM). The cells were treated with cytokines to induce differentiation; 1000 U/mL M-CSF in 10% human serum in RPMI for Macrophages, 800 U/mL GM-CSF and 500 U/mL IL-4 in 10% fetal calf serum in RPMI for Dendritic Cells.

Cytokine Activation: After 1 week of differentiation, macrophages and dendritic cells were treated with various concentrations (.1ng/mL, 1ng/mL, 10ng/mL) of IL-6 or IL-8 in serum-free RPMI, or 10ug/mL oxPAPC in 0.5% fatty-acid free BSA/serum free RPMI. Various time points were used, and post-treatment, cells and culture supernatants were harvested.

PAFAH Activity Assay: Substrate for PAFAH activity assay was 50mM 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) with .05μCi hexadecyl-2-acetyl-*sn*-glyceryl-3-phosphocholine, 1-*O*-[acetyl-(*N*)-³H] (13.5Ci/mmol) Substrate was incubated with culture supernatant for 30 min at 37°C. PAFAH activity was determined by the release of ³H-acetate and quantified via scintillation counting as described previously (4).

Western Blot: Equal quantities of protein from culture supernatants were precipitated in PBS and trichloroacetic acid at a final concentration of 20% for 30 minutes on ice. Samples were centrifuged and supernatants were discarded. Samples were washed with 300 μ L of acetone, dried under air, and then resuspended in SDS-PAGE sample buffer. PAFAH protein was detected with a rabbit antibody against the human enzyme (Cayman) and HRP-coupled anti-rabbit (Pierce). Protein from whole cell homogenates was collected in RIPA buffer and analyzed for Stat3 and phospho-Stat3 with mouse antibodies and HRP-coupled anti-mouse (Cell Signaling).

Isolation of RNA and qPCR Analysis: RNA was harvested from cells using Trizol reagent (Invitrogen) and analyzed for PAFAH transcripts via Real Time PCR (qPCR) using PAFAH specific primers, 18S specific primers, and TaqMan One-Step kit from ABI on an ICycler instrument from BioRad, as described previously (4).

ELISA Analysis: Culture supernatant was harvested from cells and ELISA analysis (BD OptEIA IL6 ELISA kit from BD Bioscience) was performed by Megan Price with our thanks.

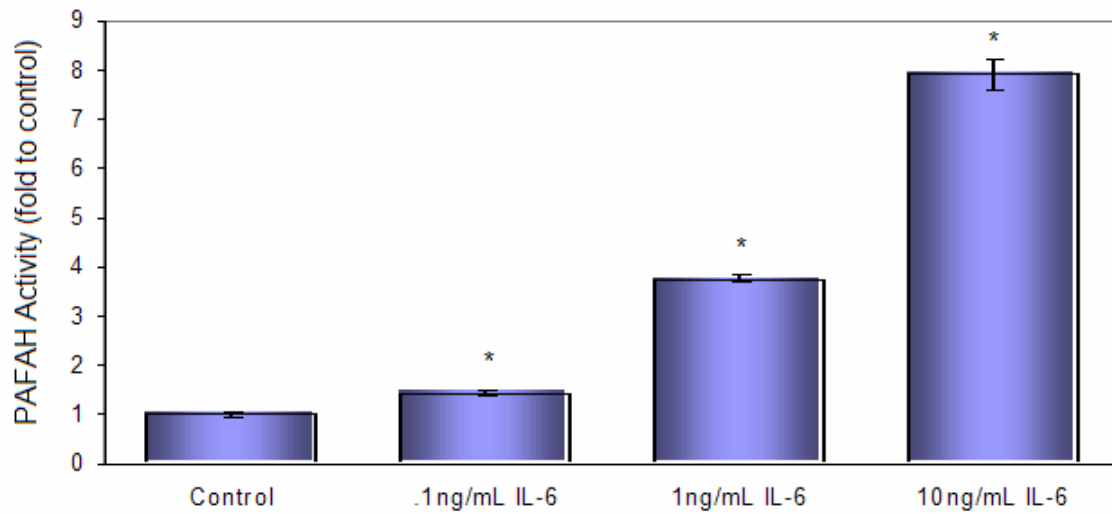
Flow Cytometry Analysis: FcR blocking reagent (Miltenyi Biotec) was incubated with 10^5 cells for 10 minutes. Cells were incubated with PE-labeled IL-6R antibody or isotype matched control (BD Pharmingen) for 30 minutes before thorough washing in PBS. Stained cells were analyzed using a FACScan (Becton Dickinson) and data were analyzed using WinMDI. Data obtained and magnanimously shared by Rachael Griffiths.

Statistical Analysis: Data are shown from a single representative experiment done in triplicate, and experiments are $n \geq 3$ unless otherwise noted. Statistical significance determined using the student's t-test, $p < 0.05$.

Results

IL-6 Induces PAFAH Secretion and Activity in Dendritic Cells: We hypothesized that IL-6 mediates the elevation of PAFAH expression in response to oxidized phospholipids. To test one element of this hypothesis, we first treated monocytes with exogenous IL-6 to see if IL-6 does indeed induce PAFAH. Dendritic cells were treated with .1ng/mL, 1ng/mL, or 10ng/mL IL-6 in serum-free media for 16 hours. The supernatant was harvested and analyzed for PAFAH activity using a radiometric enzymatic activity assay and scintillation counting. Supernatant was also TCA precipitated and PAFAH protein was analyzed using a Western Blot. There is a dose-dependant increase in both PAFAH activity and protein in response to IL-6 treatment (Fig 6).

a.)



b.)

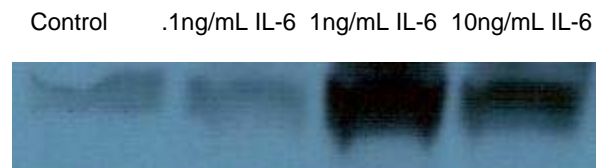


Figure 6: IL-6 Induces PAFAH in Dendritic Cells. a.) Specific Activity of PAFAH enzyme, and b.) PAFAH protein from culture supernatants. Representative replicate experiment performed in triplicate, $n = 3$, $p < .05$.

IL-6 Induces PAFAH in Dendritic Cells, but not Macrophages: To see if macrophages behave the same as dendritic cells in response to IL-6, we treated both dendritic cells and macrophages with IL-6 and compared their response. Dendritic cells and macrophages were treated with .1ng/mL, 1ng/mL, or 10ng/mL IL-6 in serum-free media for 16 hours. The supernatant was harvested and analyzed for PAFAH activity using a radiometric enzymatic activity assay and scintillation counting. PAFAH is strongly induced in dendritic cells, but the induction of PAFAH in macrophages, while sometimes significant, is weak and not reproducible (Fig 7).

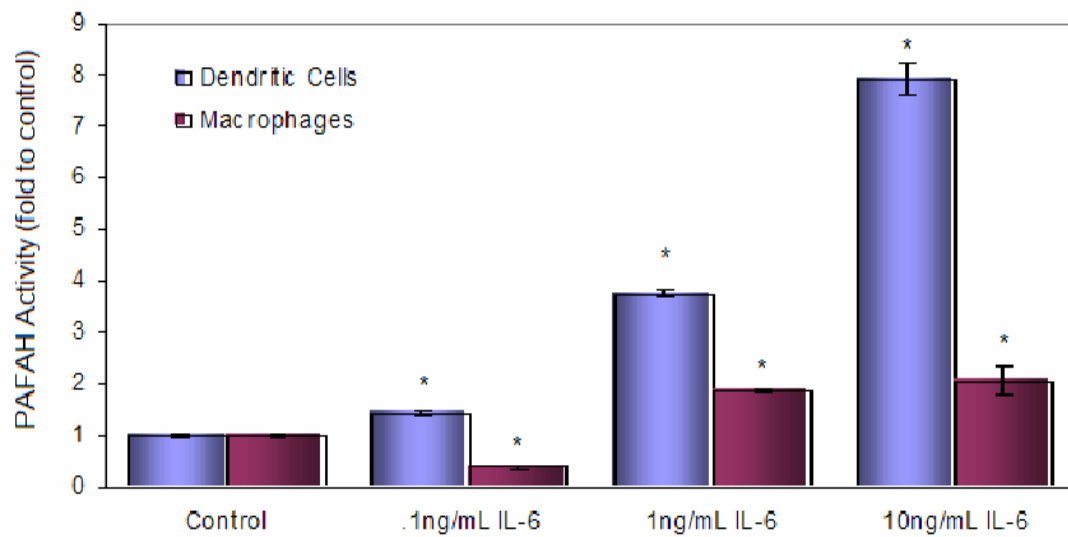
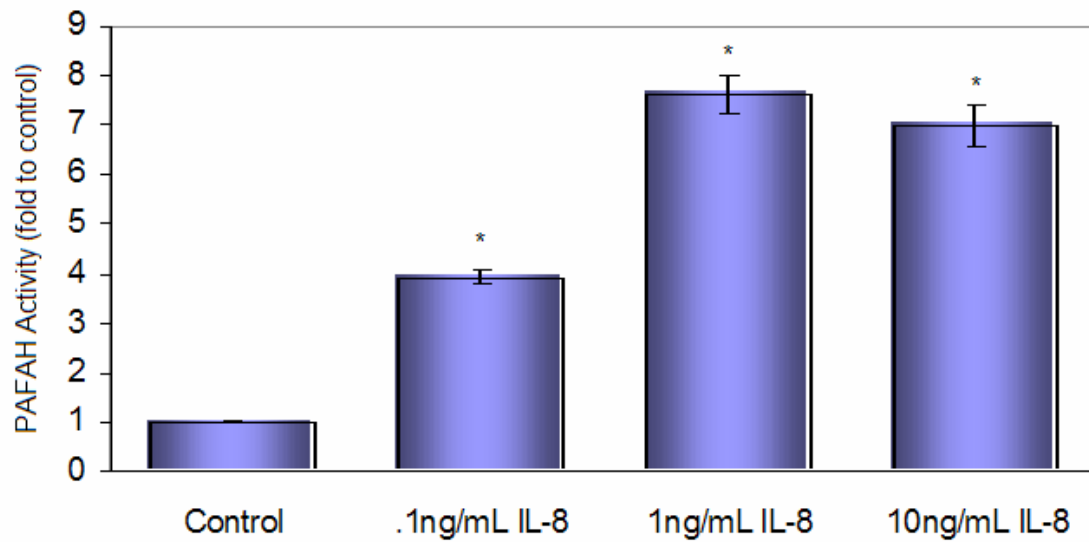


Figure 7: IL-6 Induces PAFAH Activity in Dendritic Cells, but not in Macrophages. Representative replicate experiment performed in triplicate, $n = 3$, $p < .05$.

IL-8 Induces PAFAH Secretion and Activity in Dendritic Cells: We hypothesized that IL-8 was involved in the induction of PAFAH by oxidized phospholipids. To test this, we treated dendritic cells with exogenous IL-8 to see if it induces PAFAH. Dendritic cells were treated with .1ng/mL, 1ng/mL, or 10ng/mL IL-8 in serum-free media for 16 hours. The supernatant was harvested and analyzed for PAFAH activity using a radiometric enzymatic activity assay and scintillation counting. Supernatant was also TCA precipitated and PAFAH protein was analyzed using a Western Blot. There is a dose-dependant increase in both PAFAH activity and protein in response to IL-8 treatment (Fig 8).

a.)



b.)

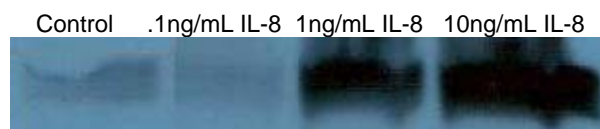


Figure 8: IL-8 Induces PAFAH in Dendritic Cells. a.) Specific Activity of PAFAH enzyme, and b.) PAFAH protein from culture supernatants. Representative replicate experiment performed in triplicate, $n = 3$, $p < .05$.

IL-8 Induces PAFAH in Dendritic Cells, but not Macrophages: To see if macrophages behave the same as dendritic cells in response to IL-8, we treated both dendritic cells and macrophages with IL-8 and compared their response. Dendritic cells and macrophages were treated with .1ng/mL, 1ng/mL, or 10ng/mL IL-8 in serum-free media for 16 hours. The supernatant was harvested and analyzed for PAFAH activity using a radiometric enzymatic assay and scintillation counting. Similar to the IL-6 trials, PAFAH is strongly induced in dendritic cells, but the induction of PAFAH in macrophages, while significant, is either weak or not reproducible (Fig 9).

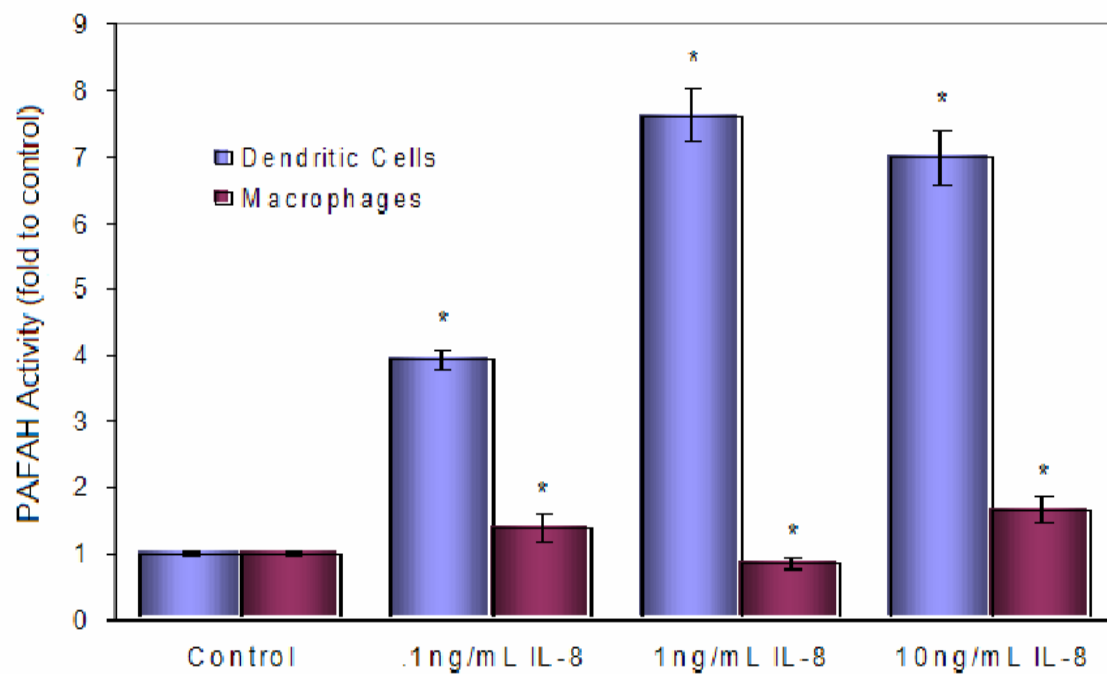
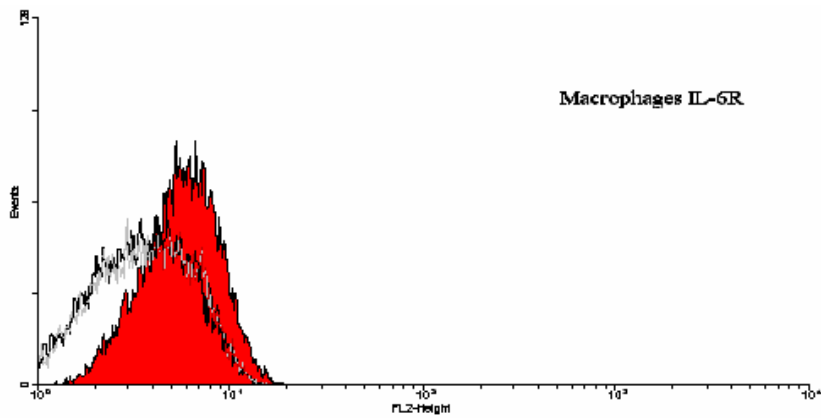


Figure 9: IL-8 Induces PAFAH Activity in Dendritic Cells, but not in Macrophages. Representative replicate experiment performed in triplicate, $n = 3$, $p < .05$.

IL-6 Receptor is More Highly Expressed in Dendritic Cells than in Macrophages:

We hypothesized that IL-6 is a key mediator of the induction of PAFAH in response to oxidized phospholipids. We found that IL-6 induces PAFAH in dendritic cells, but the response in macrophages is either blunted or absent. To test if the IL-6 receptor plays a role in this differential response, we used flow cytometry analysis to examine the cell surface expression of the IL-6 receptor in both cell types. Dendritic cells and macrophages were incubated with a Fc receptor blocking reagent, and then incubated with a PE-labeled IL-6 receptor antibody or an isotype matched control. While only a small amount of IL-6 receptor cell surface expression was detectable in macrophages, a significant increase was observed in the IL-6 receptor cell surface expression in dendritic cells (Fig 10). High expression of the IL-6 receptor in dendritic cells compared to macrophages is one possible explanation for why dendritic cells respond to IL-6 and macrophages do not.

a.)



b.)

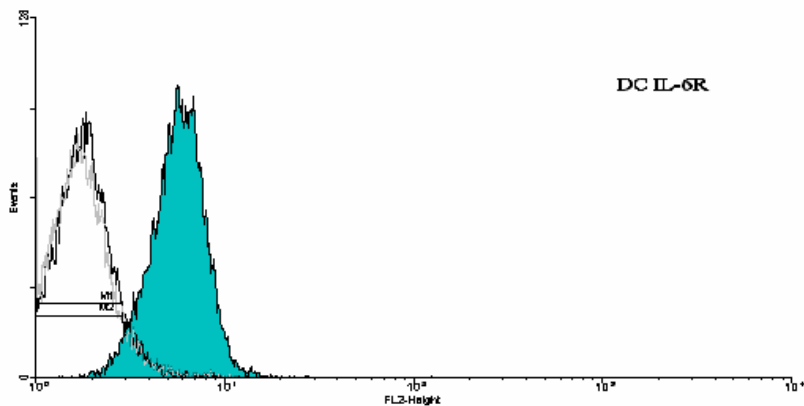
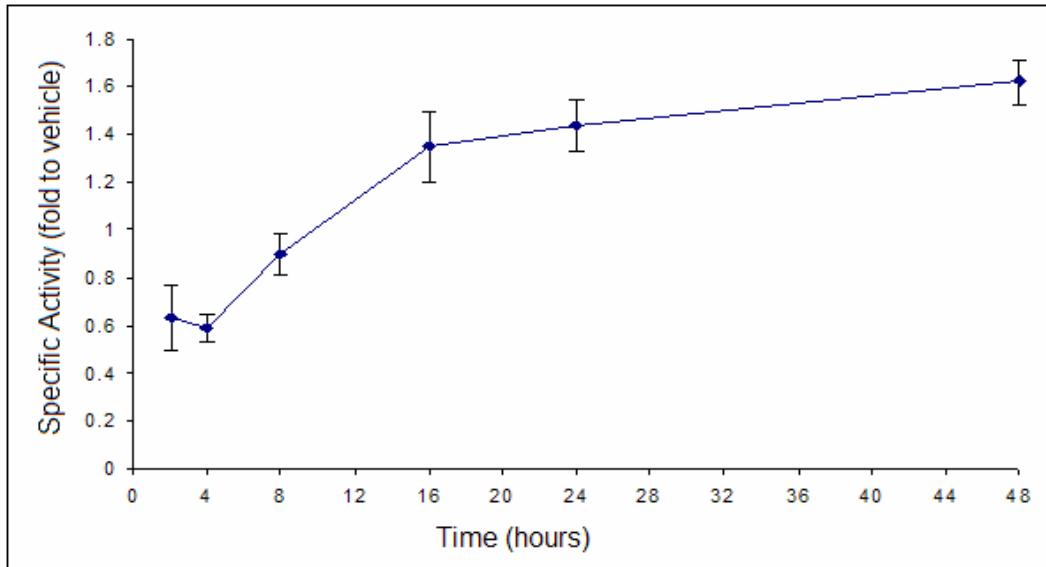


Figure 10: The IL-6 Receptor is more highly expressed in dendritic cells than in macrophages, shown by examining the cell surface expression using flow cytometry analysis. Cell surface expression of IL-6R in a.) macrophages and b.) dendritic cells. The open gray trace is a blank, negative control, and overshadowing it is the black trace, which is a negative isotype match control. The colored traces are specific for the IL-6 receptor.

Induction of PAFAH by oxPAPC is not Immediate: In our hypothesis, we suggest that IL-6 mediates the induction of PAFAH by oxidized phospholipids in an autocrine or paracrine fashion. Some time would be required for any upstream cell signaling events to take place before PAFAH induction, so we performed time-course studies with dendritic cells treated with oxPAPC and observed when PAFAH activity and expression become elevated. Dendritic cells were treated with 10ug/mL oxPAPC in 0.5% fatty-acid free bovine serum albumin (BSA) in serum-free media or treated with vehicle, 0.5% fatty-acid free BSA. The supernatant was harvested and analyzed for PAFAH activity using a radiometric enzymatic activity assay and scintillation counting. Cells were harvested in Trizol reagent and RNA was isolated and quantified with real time quantitative PCR. There is a lag phase between initial oxPAPC treatment and the appearance of PAFAH expression and activity in the treated cells compared to cells treated with vehicle. PAFAH expression is elevated at 8 hours and activity begins to appear approximately 8-16 hours post-treatment. Early time points are also noteworthy, due to modest suppression of both PAFAH activity and expression. This suppression is not an artefact, as it is reproducible. (Fig 11).

a.)



b.)

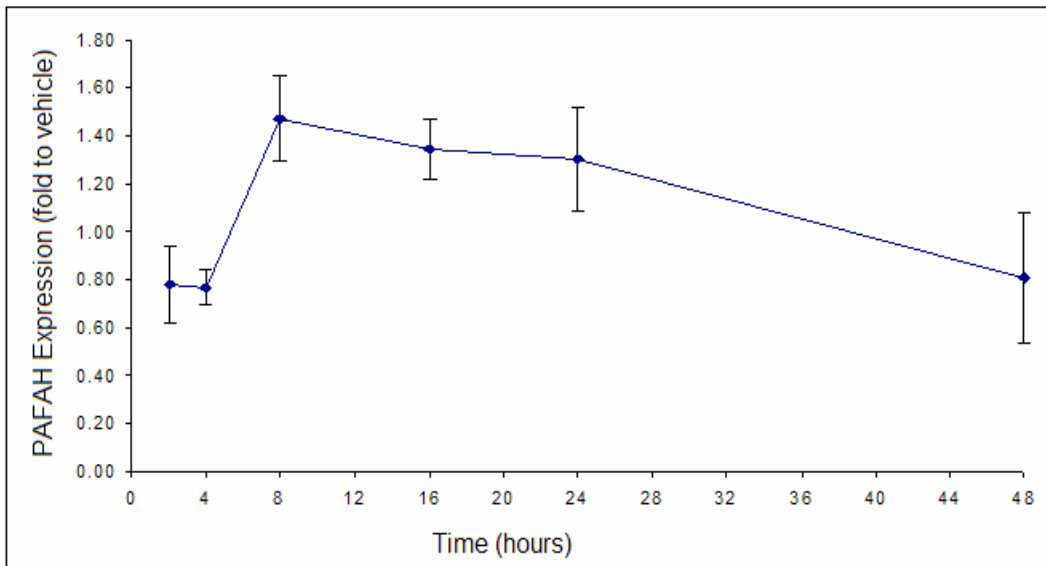


Figure 11: OxPAPC-induced PAFAH Expression and Activity follow a lag phase. Dendritic cells were treated with oxPAPC or vehicle, and PAFAH a.) activity and b.) expression were analyzed at various time points, represented here fold-to-vehicle. Representative replicate experiment performed in triplicate, $n = 3$, $p < .05$.

Ox-PAPC Induces Secretion of IL-6 in Dendritic Cells: We hypothesized that IL-6 mediates the induction of PAFAH by oxidized phospholipids in dendritic cells in an autocrine fashion. To test this element of our hypothesis, we treated dendritic cells with oxPAPC and used ELISA to observe the secretion of IL-6. Dendritic cells were treated with 10ug/mL oxPAPC in 0.5% fatty-acid free BSA in serum-free media or vehicle. The supernatant was harvested and analyzed for secreted IL-6 using ELISA. At 4 hours, induction of IL-6 in the oxPAPC treated cells is increased compared to the vehicle treated cells. Interestingly, suppression of IL-6 secretion is observed at early time points, similar to PAFAH activity and expression (Fig 12).

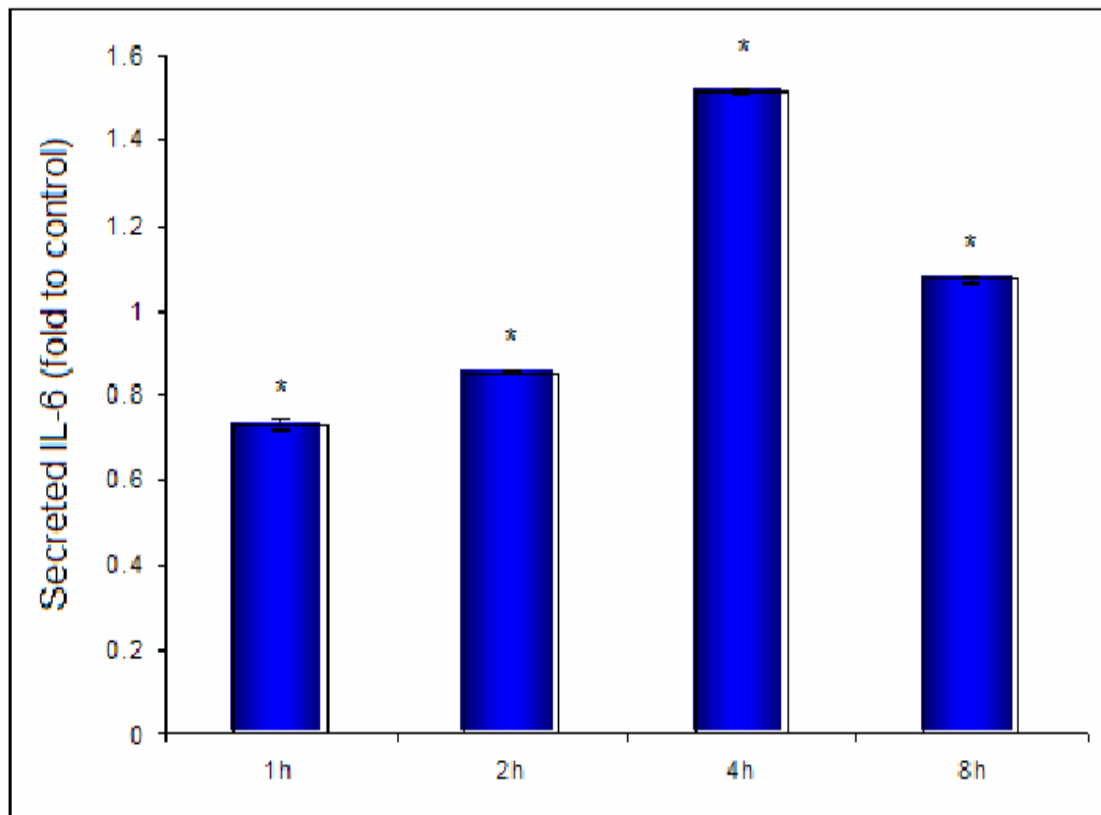


Figure 12: OxPAPC-induces secretion of IL-6 in Dendritic Cells. Dendritic cells were treated with oxPAPC or vehicle and culture supernatant was analyzed at various time points for the presence of secreted IL-6 using ELISA, represented here fold to control. n = 1, p < .05.

OxPAPC Treatment Activates Stat3 in Dendritic Cells: We hypothesized that IL-6 mediates the induction of PAFAH by oxidized phospholipids. Stat3 is a classic target of IL-6, thus we used Western Blot analysis to observe the activation of Stat3 in dendritic cells in response to treatment with oxPAPC. Dendritic cells were treated with 10ug/mL oxPAPC in 0.5% fatty-acid free BSA in serum-free media or vehicle. At various time points, whole cell homogenates were harvested in RIPA buffer. The activated form of Stat3, tyrosine phosphorylated Stat3, was analyzed by Western Blot analysis. At 4 hours, the presence of phospho-Stat3 in the oxPAPC treated cells is increased compared to the vehicle treated cells (Fig 13).

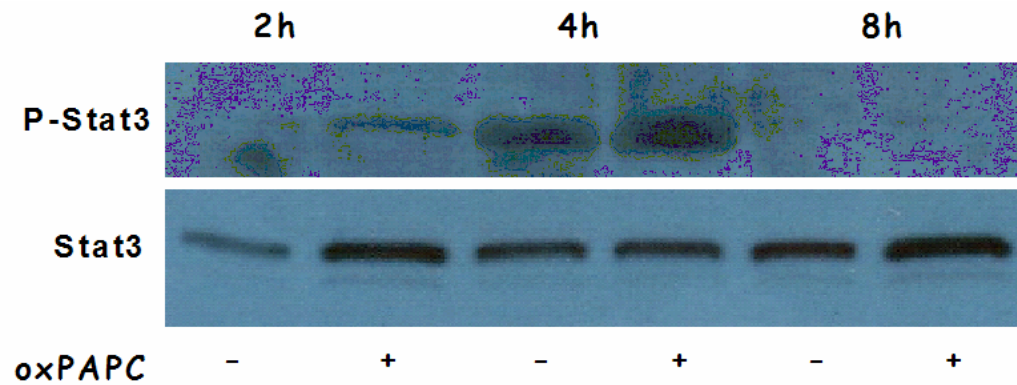


Figure 13: OxPAPC treatment activates Stat3 in Dendritic Cells. Dendritic cells were treated with oxPAPC or vehicle and whole cell homogenates were collected at various time points in RIPA buffer and analyzed for phospho-Stat3 and total Stat3. $n = 1$.

IL-6 Induction of PAFAH Activity is Present by 8 hours: We hypothesized that IL-6 mediates the elevation of PAFAH expression in response to oxidized phospholipids. To determine when this elevation occurred, we treated dendritic cells with exogenous IL-6. Dendritic cells were treated with 10ng/mL IL-6 in serum-free media and culture supernatant was harvested at various time points. The supernatant was then analyzed for PAFAH activity using a radiometric enzymatic activity assay and scintillation counting. PAFAH enzymatic activity was observed to be elevated by approximately 8 hours post IL-6 treatment (Fig 14).

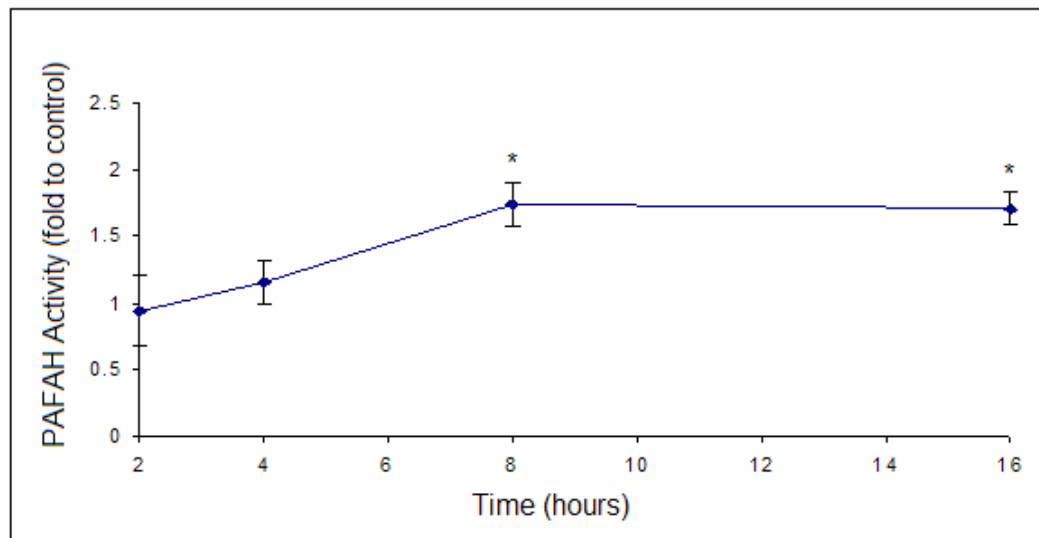


Figure 14: IL-6 Induces PAFAH Activity in Dendritic Cells by 8 hours. Specific Activity of PAFAH enzyme was quantified from culture supernatants. Representative replicate experiment, $n = 2$, $p < .05$.

Discussion

We hypothesized that in atherosclerosis, oxPAPC induces dendritic cells to express increased levels of PAFAH by binding the EP2 receptor. This causes an increase in IL-6 secretion which autocrinely activates the cell of origin or paracrinely activates adjacent cells. IL-6 then activates Stat3, which drives PAFAH expression and secretion. Subsequently, Stat3 drives IL-8 expression which then augments the PAFAH response through an unknown mechanism.

Here, we have shown that IL-6 and IL-8 robustly induce PAFAH expression and activity in dendritic cells but not in macrophages. We have shown that this differential response may in part be due to the decreased expression of cell surface IL-6 receptor in macrophages. We have conducted time-course studies of oxPAPC-induced activation of PAFAH in dendritic cells, showing that PAFAH expression is elevated 8 hours post-treatment and that PAFAH activity begins to rise approximately 8-16 hours post-treatment, demonstrating that a lag phase precedes activation, suggesting other cellular signaling pathways are required to be activated first. We have shown that IL-6 is being secreted from dendritic cells activated with oxPAPC, and that this increase in IL-6 is most apparent at 4 hours. We have also shown that Stat3 is being activated in dendritic cells treated with oxPAPC, also at 4 hours post-treatment.

These time course data support our model, illustrating that IL-6 and Stat3 are being activated approximately 4 hours post-treatment with oxPAPC, followed by an increase in PAFAH expression at 8 hours, and finally an increase in PAFAH activity between 8 and

16 hours (Fig 15). The IL-6 time course data also adhere to this model. IL-6-induced PAFAH activity was not apparent until 8 hours post-treatment, suggesting that if IL-6 secretion is at its highest around 4 hours post-oxPAPC treatment, then perhaps 8 hours after the secretion of IL-6, PAFAH activity increases. This fits into our time course data of PAFAH activity being elevated between 8 and 16 hours post-oxPAPC treatment.

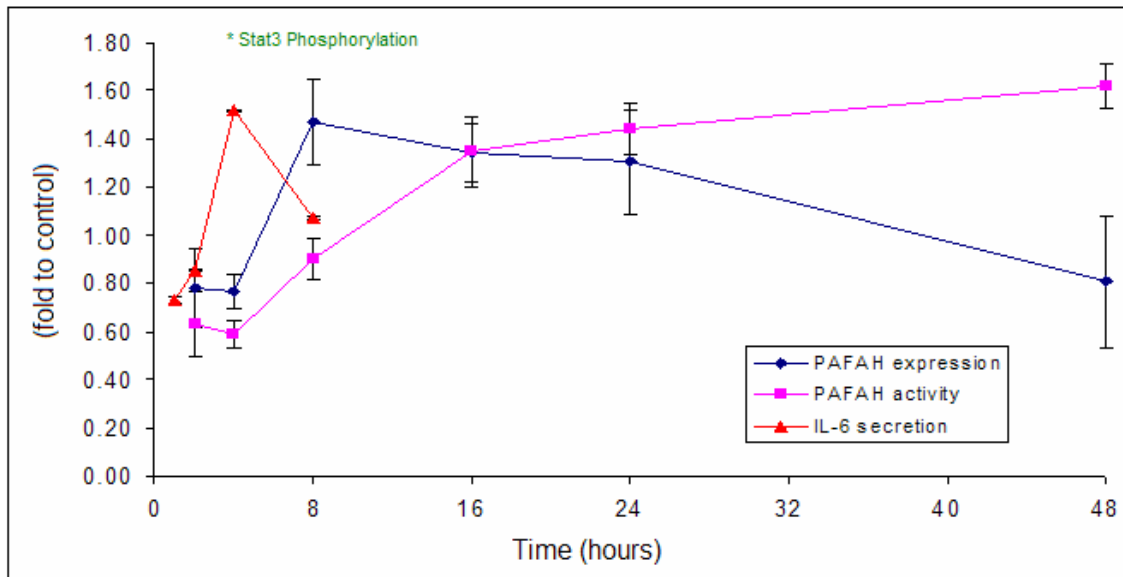


Figure 15: Time-course showing changes in IL-6 secretion, Stat3 activation, and PAFAH expression and activity. IL-6 secretion increases at 4 hours, Stat3 is then phosphorylated, followed by an increase in PAFAH expression, and finally PAFAH activity. Previous IL-6 treatment time-course experiments have shown an increase in PAFAH activity after 8 hours, supporting this data.

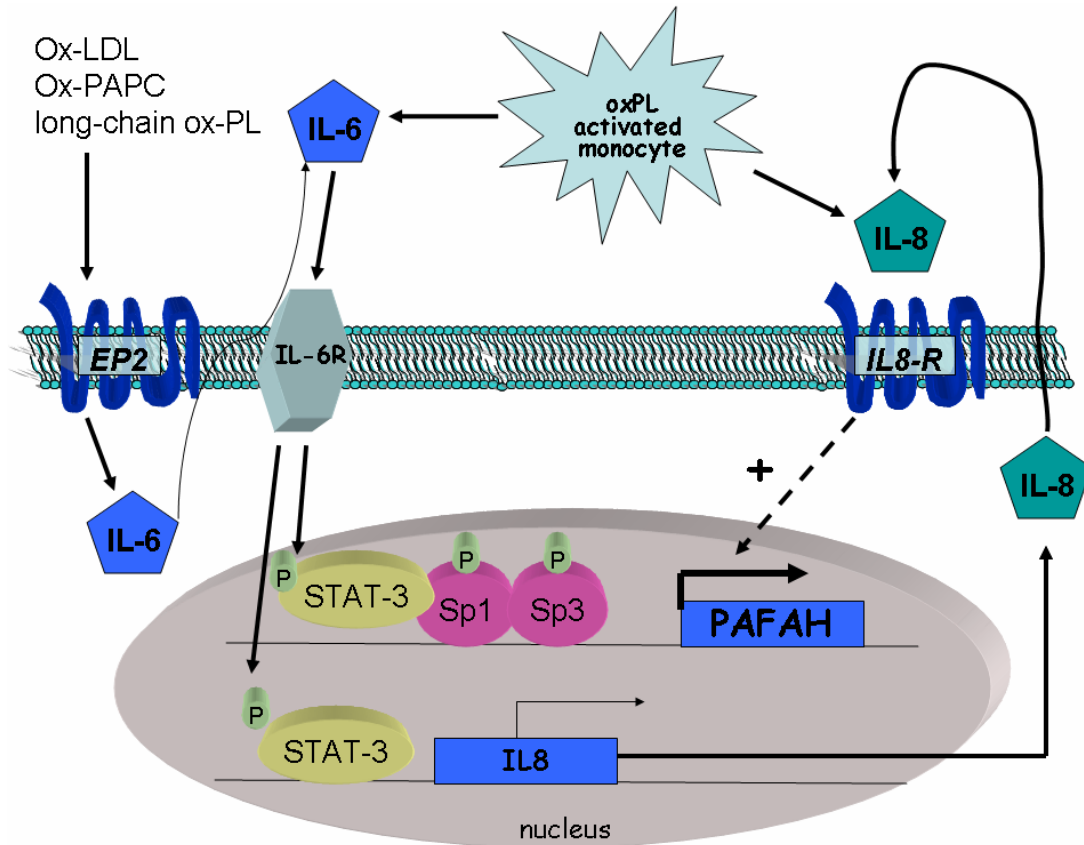


Figure 16: Theoretic Model. We hypothesize that oxidized phospholipids induce PAFAH by binding the EP2 receptor. This interaction induces IL-6 which further activates dendritic cells in an autocrine fashion. IL-6 activates STAT-3, a transcription factor that not only induces PAFAH, but also induces IL-8 which further augments PAFAH induction by binding its receptor.

While our evidence heavily supports our model, further studies must be conducted to determine whether IL-6 is actually the mediating factor by which oxPAPC activates PAFAH in dendritic cells. We have shown that IL-6 secretion is not only upregulated in response to oxPAPC, but that IL-6 induces PAFAH activity. To confirm that IL-6 is the link between oxPAPC and PAFAH induction, blocking IL-6 in oxPAPC treated cells and abolishing the induction of PAFAH would neatly illustrate our model. While siRNA is not a favorable option due to the difficulty with transfection in primary monocytes, a neutralizing antibody against IL-6 or the IL-6 receptor should be more feasible. Furthermore, other studies to confirm our model must be conducted. As we have shown the increase in secretion of IL-6 from dendritic cells and the increase of the IL-6 receptor on the cell surface of dendritic cells compared to macrophages, we must also study the secretion of IL-8 and the cell surface expression of the IL-8 receptor. We are also currently performing quantitative real time PCR for IL-6 and IL-8 transcripts to quantify any upregulation on the expression level. Furthermore, more time-course studies must be conducted to determine when IL-8 is becoming upregulated in response to oxPAPC and when that has an effect on PAFAH upregulation. If our hypothesis regarding IL-8 is correct, secretion of IL-8 would be detectable further down the pathway, between 16-24 hours. While it is not surprising that PAFAH enzymatic activity is still elevated at 24 hours, it is interesting that PAFAH expression is still elevated at 24 hours despite any cellular degradation and turnover of mRNA, suggesting that perhaps this is an effect of IL-8 further augmenting the induction of PAFAH.

In addition, more studies must be conducted to explain the early suppression of PAFAH activity, PAFAH expression, and IL-6 secretion. Our lab has shown that PAF and other short-chain PAF-like lipids can suppress PAFAH induction. One possibility may be that oxidatively-fragmented short-chain lipids present in oxPAPC are signaling through the PAF receptor or an unidentified receptor. To determine if this is true, a time course study would need to be conducted in which we treat dendritic cells with a PEIPC-rich fraction and see if the early suppression is abolished or remains.

In conclusion, our data supports our model that PAFAH levels are induced by proinflammatory mediators that have already been associated with the progression of atherosclerotic lesions, and that a clinical treatment may be developed by further elucidating the mechanism of PAFAH regulation.

Literature Cited

Literature Cited

1. Lodish, H. F., et al. 2004. Molecular Cell Biology. *W. H. Freeman and Company*
5th Edition:768-769.
2. Getz,G.S. 2005. Thematic review series: The Immune System and Atherogenesis.
Immune function in atherogenesis. *J. Lipid Res.* **46**:1-10.
3. Watson,A.D., Leitinger,N., Navab,M., Faull,K.F., Horkko,S., Witztum,J.L.,
Palinski,W., Schwenke,D., Salomon,R.G., Sha,W. et al 1997. Structural
Identification by Mass Spectrometry of Oxidized Phospholipids in Minimally
Oxidized Low Density Lipoprotein That Induce Monocyte/Endothelial Interactions
and Evidence for Their Presence in Vivo. *J. Biol. Chem.* **272**:13597-13607.
4. Leitinger,N. 2005. Oxidized phospholipids as triggers of inflammation in
atherosclerosis. *Mol Nutr. Food Res* **49**:1063-1071.
5. Li,R., Mouillesseaux,K.P., Montoya,D., Cruz,D., Gharavi,N., Dun,M.,
Koroniak,L., and Berliner,J.A. 2006. Identification of Prostaglandin E2 Receptor
Subtype 2 As a Receptor Activated by OxPAPC. *Circulation Research* **98**:642-650.
6. Fiotti,N., Giansanti,C., Ponte,E., Delbello,C., Calabrese,S., Zacchi,T., Dobrina,A.,
Guarnieri,G. 1999. Atherosclerosis and Inflammation. Patterns of cytokine
regulation in patients with peripheral arterial disease. *Atherosclerosis* **145**:51-60.

7. Boekholdt,S.M., Peters,R.J.G., Hack,C.E., Day,N.E., Luben,R., Bingham,S.A., Wareham,N.J., Reitsma,P.H., Khaw,K. 2004. IL-8 Plasma Concentrations and the Risk of Future Coronary Artery Disease in Apparently Healthy Men and Women: The EPIC-Norfolk Perspective Population Study. *Arterioscler. Thromb. Vasc. Biol.* **24**:1503-1508.
8. Stafforini,D.M., McIntyre,T.M., Zimmermann,G.A., and Prescott,S.M. 1997. Platelet-activating factor acetylhydrolases. *J. Biol. Chem.* **272**:17895-17898.
9. Elstad,M.R., Stafforini,D.M., McIntyre,T.M., Prescott,S.M., and Zimmerman,G.A. 1989. Platelet activating factor acetylhydrolase increases during macrophage differentiation. *J. Biol. Chem* **264**:8467-8470.
10. Al Darmaki,S., Schenkein,H.A., Tew,J.G., and Barbour,S.E. 2003. Differential expression of platelet-activating factor acetylhydrolase in macrophages and monocyte-derived dendritic cells. *J. Immunol.* **170**:167-173.
11. Yamada,Y., Yoshida,H., Ichihara,S., Imaizumi,T., Satoh,K., and Yokota,M. 2000. Correlations between plasma platelet-activating factor acetylhydrolase (PAF-AH) activity and PAF-AH genotype, age, and atherosclerosis in a Japanese population. *Atherosclerosis* **150**:209-216.
12. Yamada,Y., Ichihara,S., Fujimura,T., and Yokota,M. 1998. Identification of the G994--> T missense in exon 9 of the plasma platelet-activating factor acetylhydrolase gene as an independent risk factor for coronary artery disease in Japanese men. *Metabolism* **47**:177-181.

13. Blankenberg,S., Stengel,D., Rupprecht,H.J., Bickel,C., Meyer,J., Cambien,F., Tiret,L., and Ninio,E. 2003. Plasma PAF-acetylhydrolase in patients with coronary artery disease: results of a cross-sectional analysis. *J. Lipid Res.* **44**:1381-1386.
14. Packard,C.J., O'Reilly,D.S.J., Caslake,M.J., McMahon,A.D., Ford,I., Cooney,J., Macphee,C.H., Suckling,K.E., Krishna,M., Wilkinson,F.E. et al 2000. Lipoprotein-Associated Phospholipase A2 as an Independent Predictor of Coronary Heart Disease. *N Engl J Med* **343**:1148-1155.
15. Blake,G.J., Dada,N., Fox,J.C., Manson,J.E., and Ridker,P.M. 2001. A prospective evaluation of lipoprotein-associated phospholipase A(2) levels and the risk of future cardiovascular events in women. *J Am Coll. Cardiol.* **38**:1302-1306.
16. Marathe,G.K., Zimmerman,G.A., Prescott,S.M., and McIntyre,T.M. 2002. Activation of vascular cells by PAF-like lipids in oxidized LDL. *Vascular Pharmacology* **38**:193-200.
17. Leitinger,N. 2006. A rancid culprit in vascular inflammation acts on the prostaglandin receptor EP2. *Circ Res* **98**:587-589.
18. Wu,X.Q., Stafforini,D.M., and Prescott,S.M. 2003. Sp1 and sp3 transcription factors mediate basal expression of the murine plasma PAF-acetylhydrolase gene in macrophages. *FASEB J.* **17**:A1010.
19. Memon,R.A., Fuller,J., Moser,A.H., Feingold,K.R., and Grunfeld,C. 1999. In vivo regulation of plasma platelet-activating factor acetylhydrolase during the acute phase response. *Am J Physiol Regul Integr Comp Physiol* **277**:R94-103.

20. Wu,X., Zimmerman,G.A., Prescott,S.M., and Stafforini,D.M. 2004. The p38 MAPK Pathway Mediates Transcriptional Activation of the Plasma Platelet-activating Factor Acetylhydrolase Gene in Macrophages Stimulated with Lipopolysaccharide. *J. Biol. Chem.* **279**:36158-36165.
21. Han,C., Demetris,A.J., Stolz,D.B., Xu,L., Lim,K., and Wu,T. 2006. Modulation of Stat3 activation by the cytosolic phospholipase A2alpha and cyclooxygenase-2-controlled prostaglandin E2 signaling pathway. *J Biol Chem.* **281**:24831-24846.
22. Yeh,M., Gharavi,N.M., Choi,J., Hsieh,X., Reed,E., Mouillesseaux,K.P., Cole,A.L., Reddy,S.T., and Berliner,J.A. 2004. Oxidized phospholipids increase interleukin 8 (IL-8) synthesis by activation of the c-src/signal transducers and activators of transcription (STAT)3 pathway. *J. Biol. Chem.* **279**:30175-30181.

VITA

Muralikrishna Mukkamala was born June 29, 1983 in Charleston, West Virginia. He attended George Washington High School and did his undergraduate work at George Washington University in Washington, DC. There, he received a B.A. in Chemistry and minors in Biology and History. After doing research at the National Institute of Health, the School of Medicine at George Washington University, and the Charleston Area Medical Center Research Institute, he attended Virginia Commonwealth University in Richmond, Virginia, seeking a Masters of Science degree in the department of Biochemistry and Molecular Biology, concentrating on the regulation of lipid metabolism. While there, he won a Travel Award at the 42nd Annual Southeastern Regional Lipid Conference. He is now beginning medical school at the Joan C. Edwards School of Medicine at Marshall University in Huntington, West Virginia.